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Single Laboratory Evaluation of the Hydrogen Oxidation Soil Bioassay

EG and G Idaho, Inc., Idaho Falls

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SINGLE LABORATORY EVALUATION OF THE HYDROGEN OXIDATION SOIL BIOASSAY

by

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16. ABSTRACT

The Hydrogen Oxidation Soil Bioassay was single laboratory tested as a potential method for monitoring hazardous wastes and hazardous waste sites. The bioassay is based on the rate of hydrogen consumption by soil microorganisms. Oxidation of hydrogen to water is inhibited when various pollutants are present in the soil and the rate of this reaction can be used as an indication of potential hazard to the soil ecosystem.

The single laboratory evaluation included ruggedness testing, a determination of method sensitivity and precision, and tests to determine the limits of reliable measurement. Aqueous solutions of mercuric chloride were used as sample material during the evaluation. Some preliminary tests were also conducted using both organic compounds and actual hazardous waste samples.

The bioassay was found to be "rugged" in the sense that modest procedural variations did not produce an altered test result. The method's capability for precision, expressed as a CV of 7.8 percent, was determined by conducting 10 seperate bioassays using the same concentration of mercuric chloride. Within a mercury concentration range of 10 ppm to 150 ppm, the technique was capable of distinguishing between concentration differences of 25 ppm. The limits of reliable measurement were established at 10 ppm and 750 ppm mercury when mercuric chloride solutions are used as sample material. The complete Hydrogen Oxidation Soil Bioassay protocol, the results of chemical analyses conducted on actual samples that were used during the single laboratory test, and the preliminary bioassay responses to different types of sample material are included in the project report.

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ABSTRACT

A single laboratory evaluation of the Hydrogen Oxidation Soil Bioassay was conducted following guidelines established by the Environmental Monitoring Systems Laboratory, Las Vegas, (EMSL-LV) for single laboratory testing of biological methods (EPA-600/S4-83-056). The evaluation included "ruggedness" testing, a determination of method sensitivity and precision, and tests to determine the limits of reliable measurement. Since there was no "true value" or "true response" to a reference sample material, the methods capability for bias (systematic error) was not determined. Preliminary test results confirmed that the assay was responsive to aqueous solutions of Hg, Ag, and Cd, to metal combinations, to phenolic compounds, and to actual hazardous waste site material (i.e., metals added to waste site material to provide a more realistic sample matrix).

The method's single laboratory capability for ruggedness, precision, sensitivity, and limits of reliable measurement were determined using aqueous solutions of mercuric chloride as sample material. The bioassay was found to be "rugged" in the sense that modest procedural variations did not produce an altered test result. The method's capability for precision, expressed as a CV of 7.8 percent, was determined by conducting 10 separate assays using the same concentration of mercuric chloride. Within a mercury concentration range of 10 to 150 ppm, the technique was capable of distinguishing between concentration differences of 25 ppm. Limits of reliable measurement were established at 10 ppm and 750 ppm of mercury. The evaluated method protocol, and some recommendations for further testing, are included in this report.

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INTRODUCTION

The Hydrogen Oxidation Soil Bioassay has been single laboratory tested as a potential method for monitoring hazardous wastes and hazardous waste sites. A single laboratory test should establish the data quality that can be achieved within a single laboratory, it should provide a basis for deciding whether or not the method merits collaborative testing, and it should more clearly define a method's potential for inclusion as part of an operational monitoring network. The current evaluation, based on the EMSL-LV guidelines for the single laboratory testing of biological methods (McKenzie and Olsson, 1983), included ruggedness testing, a determination of method precision and sensitivity, and tests to determine the limits of reliable measurement. Since there was no "true value" or "true response" to a reference sample material, the method's capability for bias (systematic error) was not determined.

This bioassay is based on the rate of hydrogen consumption by ubiquitous soil microorganisms (McFarlane, 1976; McFarlane, et al., 1978; McFarlane, et al., 1979). Oxidation of hydrogen to water is inhibited when various pollutants (the method is responsive to liquid, solid, and gaseous compounds) are present in the soil and the rate of this reaction can be used as an indication of potential hazard to the soil ecosystem (Rogers and McFarlane, 1982). The technique has been evaluated as a laboratory test and assumes, when the procedure is being routinely used, that sample material (e.g., actual hazardous waste material, leachate, analytical fractions of hazardous waste material, water samples that potentially contain waste site chemicals, etc.) would be sent to the assay laboratory. Potentially, soil from a hazardous waste site area could also be used as test material, but the method has not been evaluated for this type of application.

CONCLUSIONS

The Hydrogen Oxidation Soil Bioassay was single laboratory tested as a potential method for use in hazardous waste monitoring networks. The bioassay was found to be "rugged" in the sense that modest procedural variations did not produce an altered test result. The method's capability for precision, expressed as a CV of 7.8 percent, was determined by conducting 10 separate assays using the same concentration of mercuric chloride. Within a mercury concentration range of 10 to 150 ppm, the technique was capable of distinguishing between concentration differences of 25 ppm. Limits of reliable measurement were established at 10 ppm and 750 ppm of mercury. The single laboratory tested method protocol has, of course, also been prepared. Before this bioassay can be recommended for collaborative testing, it will be necessary to conduct portions of the single laboratory test again using sample material that more realistically simulates either a hazardous waste site leachate, or an analytical fraction of actual hazardous waste material. However, results from this evaluation suggest that this terrestrial monitoring technique should ultimately be a candidate method for collaborative testing and should be of subsequent benefit to a hazardous waste monitoring network.

METHODS

The complete method protocol for the Hydrogen Oxidation Soil Bioassay is provided in Appendix A. Soil used for the bioassay was a Calico series fine sandy loam (Aquic Xerofluent) from southeastern Nevada (53.9 percent sand, 10.8 percent clay, 1.3 percent organic carbon; 12.7 meg/100 g cation exchange capacity; pH 8.6). After collection, it was air dried to a constant weight, sieved to pass a 0.66-mm screen, and stored in plastic bags at room temperature. Other studies have shown that hydrogen consumption is not altered after one year storage (Conrad and Seiler, 1981; Rogers and McFarlane, 1982). Chemicals whose toxicity was evaluated by the method were all reagent grade and included mercuric nitrate [$Hg(NO_3)_2$], mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), cadmium nitrate [$Cd(NO_3)_2$], cadmium chloride ($CdCl_2$), pyrocathechol, m-chlorophenol, and p-chlorophenol. All testing was done with aqueous solutions of these compounds. Qualitative and quantitative chemical analyses were conducted on the laboratory prepared treatment solutions and on the amended soil to verify chemical concentrations and to screen for possible contaminants. Samples collected from an actual hazardous waste site lagoon were also used as sample material during part of the bioassay evaluation. The hazardous waste site samples were also chemically analyzed (Table 1).

The bioassay protocol (Appendix A) was strictly followed during the evaluation. This procedure is briefly outlined in Figure 1.

An index analogous with the LC50 is used to compare the relative effect that compounds have on the oxidation of hydrogen. The LC50 is the concentration of a particular compound which results in lethality to 50 percent of the test organisms. Because the hydrogen oxidation test measures the rate of a chemical reaction rather than lethality, the term $P_2$50 is used to identify that concentration of a compound (ppm) that reduces the reaction rate (P_2) by 50 percent. For this study the term ppm, unless otherwise specified, is defined as µg test compound/g soil.

The $P_2$50 term is obtained from a linear regression line generated from a plot of the ratio (as a percent) of a P_{2c} (rate of hydrogen oxidation in untreated soil) and at least three P_{2t} (rate of hydrogen oxidation in treated soil) values versus the log concentration of the treatment. The calculated concentration which results in a P_{2t} value that is 50 percent of the P_{2c} is designated as the treatment $P_2$50 value. Because this value is derived by linear regression, the square of the correlation coefficient(r^2) is also reported. This coefficient indicates the strength of the linear relationship of the plotted data from which the $P_2$50 is determined.

TABLE 1. MAJOR COMPONENTS OF HAZARDOUS WASTE SITE MATERIAL USED DURING THE METHOD EVALUATION*

Semivolatile Organics (µg/L)	Volatile Organics (µg/L)
phenol	trichloroethylene
2-nitrophenol	benzene
benzoic acid	toluene
pentachlorophenol	methylene chloride
alpha-BHC	
beta-BHC	
gamma-BHC	
delta-BHC	Inorganics (mg/L)
fluoranthene	
dibenzofuran	aluminum
trimethylnaphthalene	copper
benzo(c)fluoranthene	iron
benzo(a)pyrene	sodium
di-n-butylphthalate	nickel
2-6-dinitrotoluene	selenium
1,2-benzene dicarboxylic acid	zinc

*Hazardous waste sample analysis conducted by Acurex Corporation, Mountain View, California 94039.

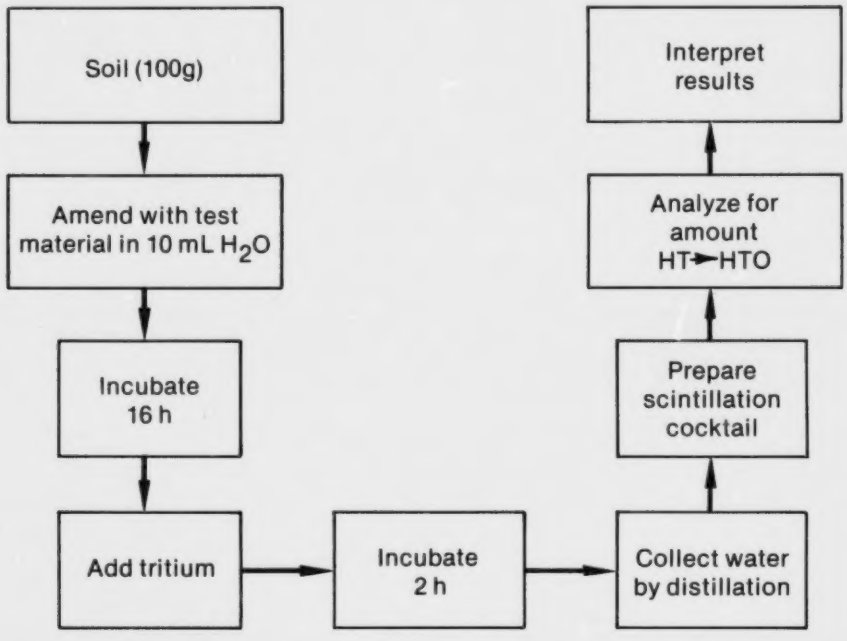


Figure 1. Outline of procedures for the Hydrogen Oxidation Soil Bioassay.

RESULTS

PRELIMINARY TESTING

This portion of the study was conducted to determine the response that the Hydrogen Oxidation Bioassay would have to toxic chemicals having a similar concentration range and similar sample matrix as might be encountered when using hazardous waste samples. Other work had indicated that the bioassay was sensitive to the effects of the heavy metals Hg, Ag, and Cd and some organics (Rogers and McFarlane, 1982). These metals as well as phenolic compounds apparently act as enzyme inhibitors (Williamson and Johnson, 1981). All of these chemicals are commonly found in industrial effluents (Sittig, 1981). In addition, the test response to these chemicals was determined when they were added to actual hazardous waste site material.

Initially, the nitrate (NO_3) forms of Hg, Ag, and Cd were tested. It was determined that Hg and Ag had $P_2$50 values of less than 100 ppm while the one for Cd was over 100 ppm (see Table 2, actual plots of the data for all tested compounds are found in Figures 2-4). In addition, the chloride (Cl) compounds of Hg and Cd (AgCl was not used because it is not soluble in H_2O at the working concentrations) were evalutated. The $P_2$50 value for $HgCl_2$ was found to be comparable with that of $Hg(NO_3)_2$ while $CdCl_2$ had a value almost seven fold greater than $Cd(NO_3)_2$. The cause of the apparent difference between the two Cd compounds was not pursued.

In addition to testing individual metals, an effort was made to determine if there were synergistic or antagonistic interactions from mixtures of the metals. Test compounds containing equal concentrations of either all three metals as NO_3 or Hg and Cd as Cl were applied to the soil. From the resulting data, it was found that the $P_2$50 values for the mixtures were not substantially different from the individual values of $Hg(NO_3)_2$ or $HgCl_2$ (see Table 2). Apparently, neither synergistic nor antagonistic interactions were caused by the mixtures. Pyrocatechol and two isomers of chlorophenol (p and m) were the three organic compounds used as sample material. All three compounds were found to have $P_2$50 values close 1000 ppm. As in the case of the mixed metals, a $P_2$50 value similar to that of $HgCl_2$ was also found for an equal concentration mixture of $HgCl_2$ and m-chlorophenol (MCP).

The hazardous waste when applied to the soil at full strength did not result in a decrease in hydrogen oxidation and, therefore, a $P_2$50 value could not be determined. This waste was then spiked with sequential additions of $HgCl_2$, $CdCl_2$, and MCP to determine what effect the waste matrix would have on the test response (Figure 4). In contrast to the mixed metals and metal organic mixture, there appeared to be an additive toxic effect in the presence

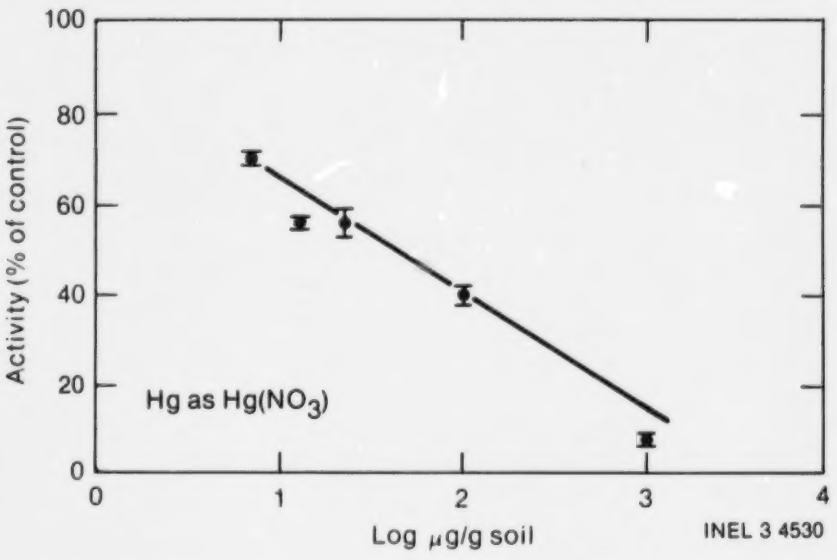
TABLE 2. $P_2 50$* AND r^2 VALUES FOR CHEMICALS AS DETERMINED BY THE HYDROGEN OXIDATION SOIL BIOASSAY

Chemical	Calculated $P_2 50$ Value	r^2 for Data Plot
$Hg(NO_3)_2$	26	0.99
$AgNO_3$	76	0.96
$Cd(NO_3)_2$	133	0.96
$HgCl_2$	36	0.99
$CdCl_2$	707	0.90
Hg, Ag, $Cd(NO_3)$	29	0.99
Hg, Cd(Cl)	52	1.00
Pyrocatechol	≈1000	not plotted†
p-Chlorophenol	≈1000	not plotted†
m-Chlorophenol	≈1000	not plotted†
$HgCl_2$ + m-chlorophenol	35	1.00
Hazardous waste (HW) 0178-222	ND#	not plotted
HW + $HgCl_2$	32	0.99
HW + $HgCl_2$ + $CdCl_2$	14	0.98
HW + $HgCl_2$ + $CdCl_2$ + m-chlorophenol	11	0.99

*$P_2 50$ is that concentration (ppm) of a compound which results in a 50 percent decrease in the oxidation of hydrogen.

†A concentration of 1000 ppm of these compounds reduced the P_2 value to between 40 and 50 percent. Increased concentrations were not used because the required stock solutions would exceed solubility of compounds in water.

#ND indicates that it was not possible to determine a $P_2 50$ value over a range of 10 to 10^3 ppm.



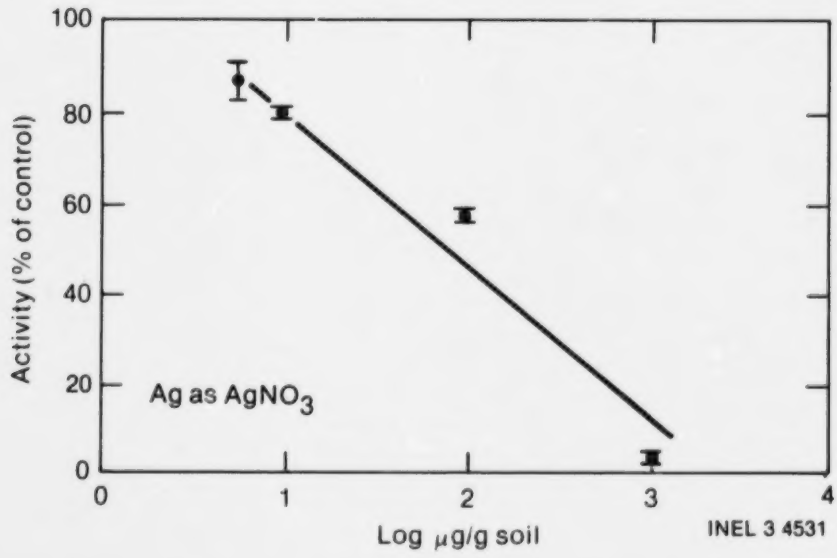
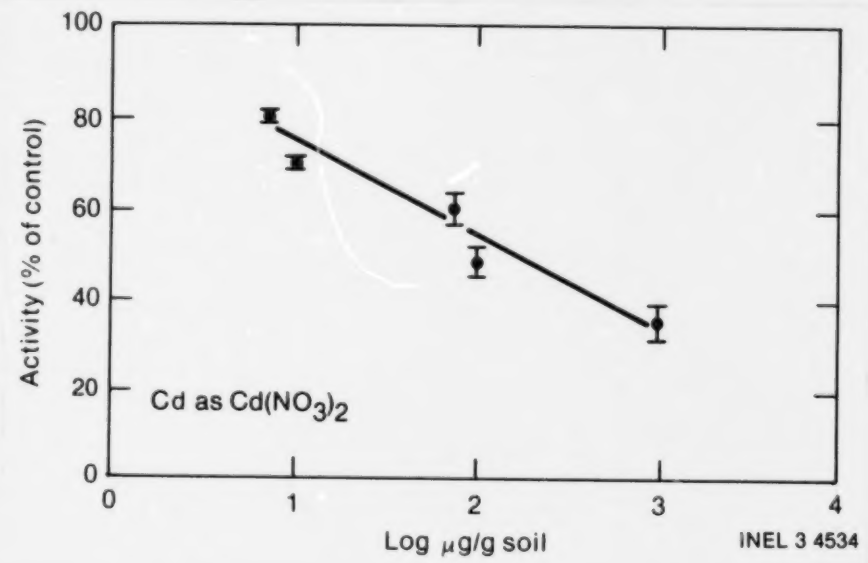
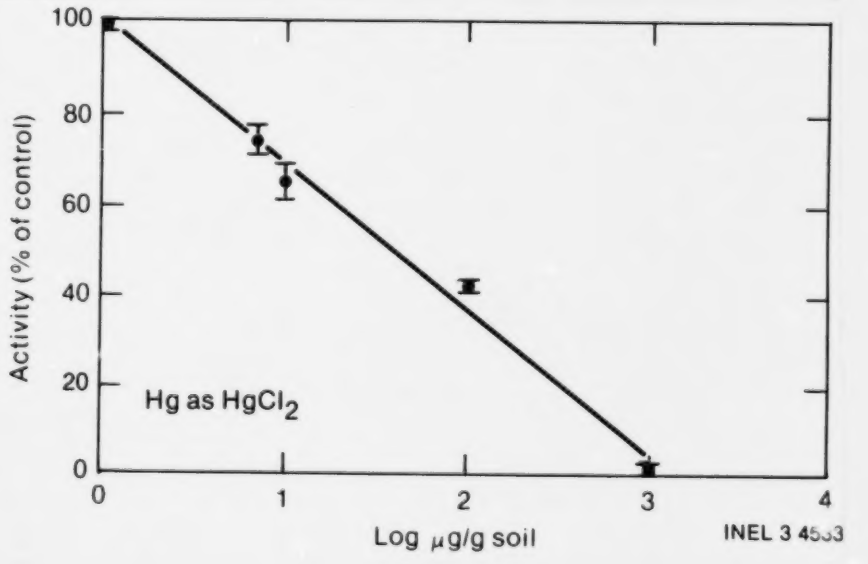


Figure 2. Percent reaction rates (P_{2t}/P_{2c}) versus toxicant concentration plotted to determine $P_2 50$ values and r^2 for monosolutions of metals using the Hydrogen Oxidation Soil Bioassay.





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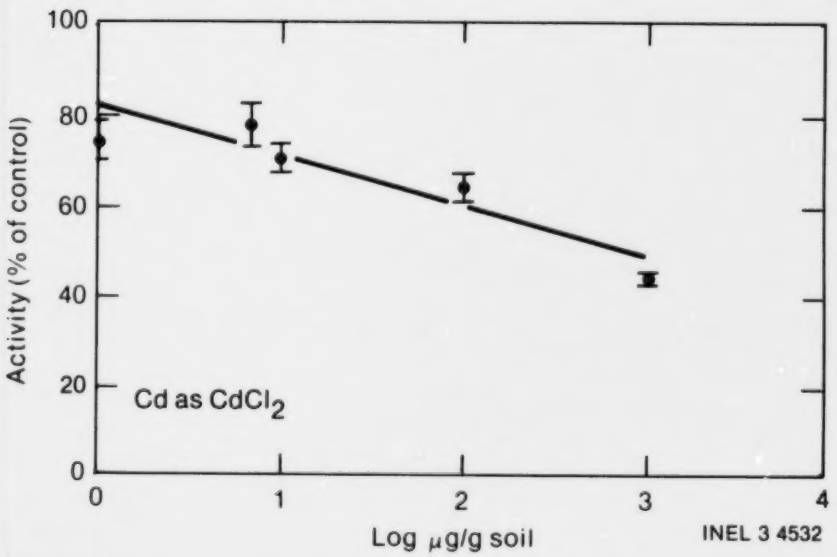

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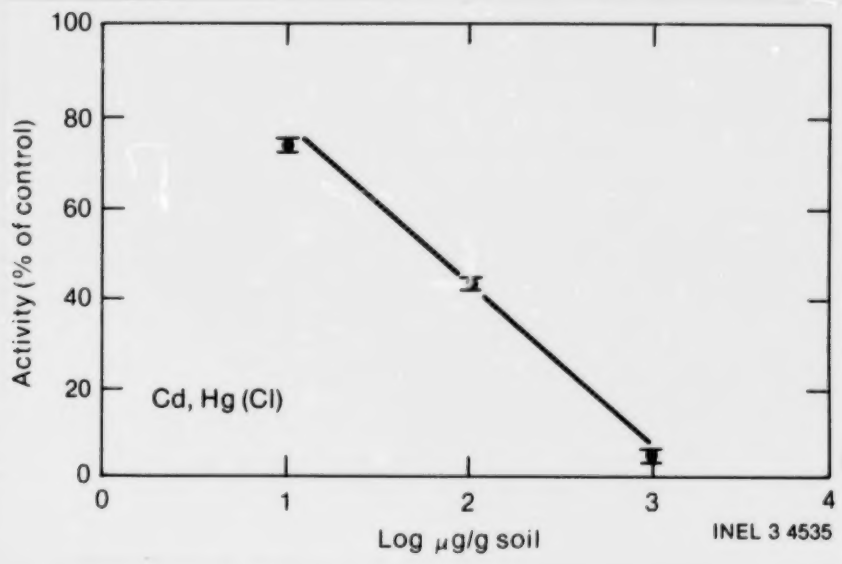


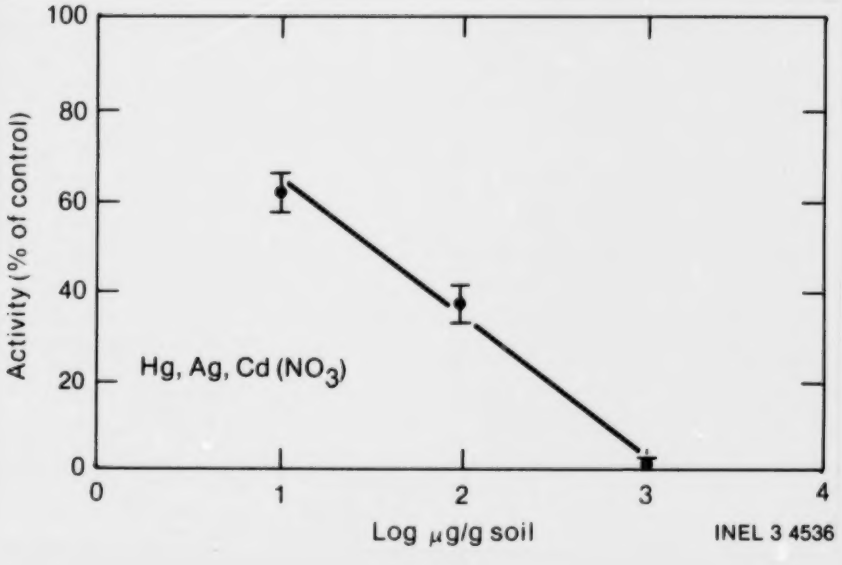
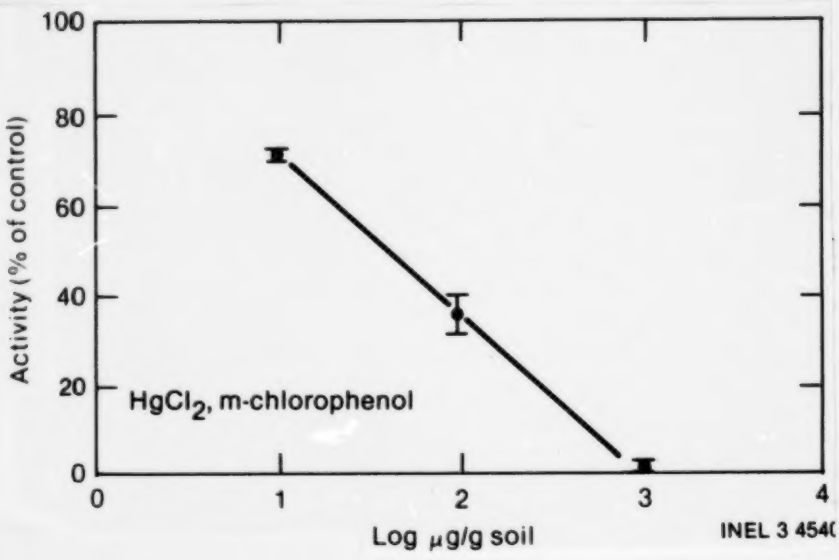


Figure 3. Percent reaction rates $(P_{2t}/P_{2c})_2$ versus toxicant concentration plotted to determine P_250 values and r^2 for defined mixtures of chemicals using the Hydrogen Oxidation Soil Bioassay.



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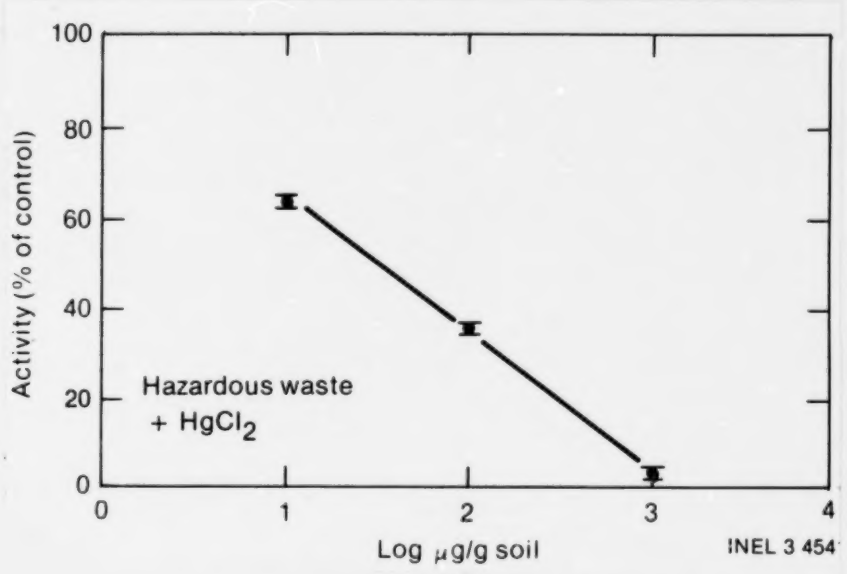

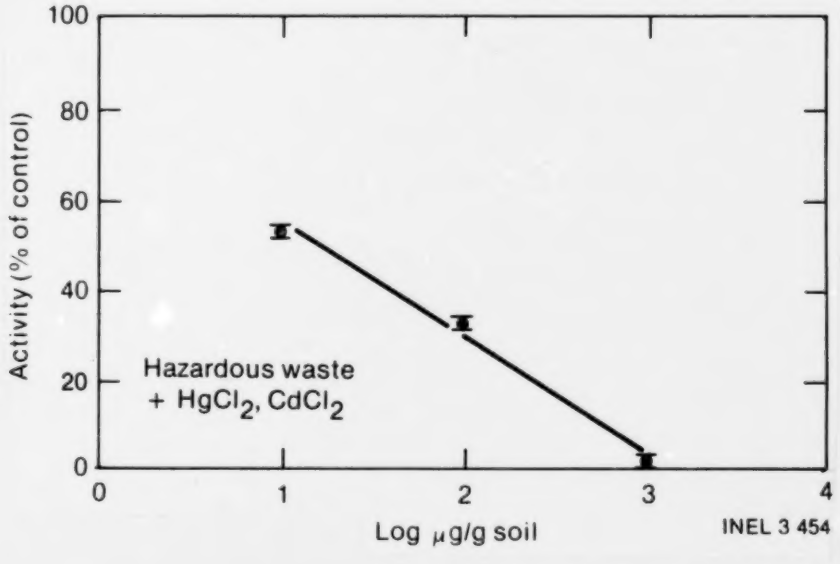

Figure 4. Percent reaction rates (P_{2t}/P_{2c}) versus toxicant concentration plotted to determine $P_2 50$ values and r^2 for mixtures of chemicals and hazardous waste using the Hydrogen Oxidation Soil Bioassay.

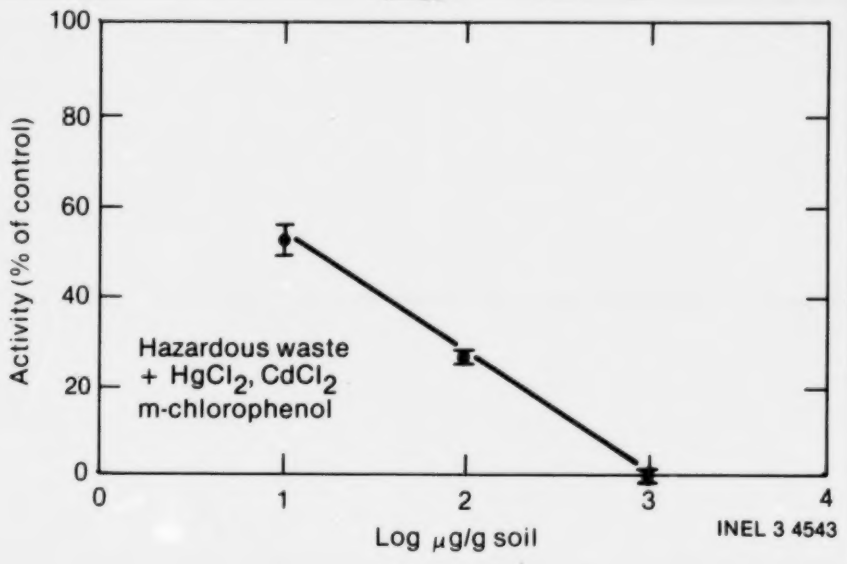

Figure 4. (Continued)

of the hazardous waste (Table 2). This was not, however, statistically substantiated since the individual $P_2$50 values were not replicated.

Aqueous solutions of mercuric chloride were selected for use as sample material during the single laboratory test, both because Hg appeared to be more toxic than Ag and Cd, and because $HgCl_2$ is more soluble than $Hg(NO_3)_2$. An aqueous solution of mercuric chloride might also be used as a much simplified example of a hazardous waste site leachate. Qualitative and quantitative analyses were conducted on all mercuric chloride sample material to confirm the chemical composition and to insure lack of contamination (Appendix B).

RUGGEDNESS TESTING

This portion of the single laboratory test determined to what extent modest procedural alterations, of a magnitude that could be made by a qualified laboratory following the written protocol, would have on the test result. A method's ability to produce an unaltered test result when subjected to minor procedural variations is an indication of method ruggedness. If the results are altered by small procedural variations, it is important to emphasize in the protocol that a specific step must be strictly followed or, in some cases, to provide more detail on any quality control steps associated with the critical procedure.

The prescribed method procedure and the corresponding procedural variations used during the ruggedness evaluation are summarized in Table 3. The seven protocol directed procedures (A-G) were chosen because they are the ones which, in our judgment, could inadvertently be altered as indicated by a-g. The protocol directed procedures and the corresponding procedural variations were then arranged into a series of eight trials. Each trial consisted of a single analysis of a single concentration of the test compound (50 ppm Hg) and a combination of procedural variations. Table 4 presents the ruggedness test design which includes abbreviations that represent the method directed procedures as well as the intentionally introduced corresponding, procedural variations (Youden, 1969). Test results from the eight trials are indicated as s-z.

The effect of each individual variable was obtained by calculating the mean oxidation value for all the treatments which contained that variation. For example, the factor A was part of the trial which produced the test results s, t, u, and v. Therefore, the mean for A can be calculated from the summation of s-v [$A = (s + t + u + v)/4$]. Combinations of analytical results required to obtain mean fractoral responses are summarized in Table 5. After tabulation of the means, the differences found between prescribed and modified variables--e.g., A-a, B-b, C-c, D-d, etc. -- were computed. If one or more variables are having an effect on the test result, their respective differences should be greater than the group of differences associated with the other variables.

Basically the procedural variations had little effect on the test result (Table 6). The modest alteration to variable G, amount of HTO distilled from soil, appears to have had the greatest effect followed by altered variables E

TABLE 3. VARIATIONS IN THE HYDROGEN OXIDATION SOIL BIOASSAY USED TO DETERMINE "RUGGEDNESS"

Item	Protocol Directed	Variation
1. Length of time a flask is purged with air both before introduction of HT and to flush out remaining HT after incubation.	A. Purge time 10 s	a. Purge time 6 s
2. Length of time soil is pre-incubated with test compound.	B. Preincubation time 16 h	b. Preincubation time 20 h
3. Length of time soil is incubated in presence of HT.	C. Incubation time 120 min	c. Incubation time 135 min
4. Amount of water, containing test compound, applied to soil.	D. Amount water 10 ml	d. Amount water 11 ml
5. Frequency of mixing soil after application of water.	E. Frequency of mixing 2 beats/s	e. Frequency of mixing 1 beat/s
6. Quantity of HTO derived from distillation which is mixed with liquid scintillation cocktail.	F. Quantity of HTO 8 ml	f. Quantity of HTO 7.9 ml
7. Amount of HTO distilled from soil.	G. Amount of HTO distillate 15 ml	g. Amount of HTO distillate 17 ml

TABLE 4. EXPERIMENTAL DESIGN FOR THE SEVEN VARIABLE "RUGGEDNESS" TEST USED IN THE SINGLE LABORATORY EVALUATION OF THE HYDROGEN OXIDATION SOIL BIOASSAY

Experiment Number	Factor Level	Analysis Result
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	a B C d e F g	w
6	a B c d E f G	x
7	a b C D e f G	y
8	a b c D E F g	z

TABLE 5. PROCEDURE USED TO DETERMINE THE AVERAGE PERCENT HT OXIDATION VALUES BASED ON ANALYTICAL DATA OBTAINED FROM "RUGGEDNESS" TESTING OF THE HYDROGEN OXIDATION SOIL BIOASSAY

Procedural Factors	Average Analytical Results
A	(s + t + u + v)/4
a	(w + x + y + z)/4
B	(s + t + w + x)/4
b	(u + v + y + z)/4
C	(s + u + w + y)/4
c	(t + v + x + z)/4
D	(s + t + y + z)/4
d	(u + v + w + x)/4
E	(s + u + x + z)/4
e	(t + v + w + y)/4
F	(s + v + w + z)/4
f	(t + u + x + y)/4
G	(s + v + x + y)/4
g	(t + u + w + z)/4

and F. This suggests that care must be taken to acquire exactly 15 ml of HTO distillate from the soil. However, the bioassay appears to be "rugged" in the sense that modest variations in method procedure would not be expected to alter the test result. No revisions were made to the method protocol.

METHOD PRECISION

Method precision was determined by conducting 10 separate assays using the same concentration of the same test material. Each of the separate determinations represented a valid test response as directed by the method protocol. Testing was conducted on alternate days and used aqueous mercuric chloride (75 ppm) as sample material. The average test response was a 61.6 percent reduction in HT oxidation with a CV of 7.8 percent.

METHOD SENSITIVITY

In the context of a single laboratory test, a method's sensitivity is defined as the method's capability to distinguish between small changes in

TABLE 6. CALCULATIONS USED TO DETERMINE THE "RUGGEDNESS" OF THE HYDROGEN OXIDATION SOIL BIOASSAY

Procedural Factors	Analytical Results	$\bar{X}$	Difference Between Method Procedure and Procedural Variation
A	44.4 + 41.2 + 29.4 + 52.1	41.8	A-a = -3.6
a	47.9 + 47.9 + 44.4 + 41.2	45.4	
B	44.4 + 41.2 + 47.9 + 47.9	45.4	B-b = 3.6
b	29.4 + 52.1 + 44.4 + 41.2	41.8	
C	44.4 + 29.4 + 47.9 + 44.4	41.5	C-c = -4.1
c	41.2 + 52.1 + 47.9 + 41.2	45.6	
D	44.4 + 41.2 + 47.9 + 41.2	43.7	D-d = -0.6
d	29.4 + 52.1 + 47.9 + 47.9	44.3	
E	44.4 + 29.4 + 47.9 + 41.2	40.7	E-e = -5.7
e	41.2 + 52.1 + 47.9 + 44.4	46.4	
F	44.4 + 52.1 + 47.9 + 41.2	46.4	F-f = 5.7
f	41.2 + 29.4 + 47.9 + 44.4	40.7	
G	44.4 + 52.1 + 47.9 + 44.4	47.2	G-g = 7.3
g	41.2 + 29.4 + 47.9 + 41.2	39.9	

pollutant concentration. The ability of this method to distinguish between changes in Hg concentration was initially tested using one concentration greater than a reference concentration, and one lower. Ten independent analyses for each of the concentrations were then conducted. If the method can distinguish between the reference concentration and the extreme concentrations, the concentration interval is reduced. This process was repeated (during the current evaluation) until the difference between the reference concentration and the selected concentration was 25 ppm.

Response of the method (percent reduction in HT oxidation) to the concentration of Hg used in the precision test (Table 7) was used as a reference to which the effects of increased or decreased concentrations of Hg could be compared. The reference concentration for Hg was 75 ppm with 10 and 150 ppm being the extremes and 50 and 100 ppm as the reduced concentration intervals between the reference concentration and the extremes. This design made it possible to determine if the bioassay could distinguish between Hg concentration intervals of 75 ppm (65 ppm on the lowest concentration) and between intervals of 25 ppm.

Test results from the different concentrations of sample material are given in Table 8. Statistical comparisons between the reference concentration and those used as brackets were made (analysis of variance). Differences in

TABLE 7. DATA USED TO CALCULATE THE PRECISION OF THE HYDROGEN OXIDATION SOIL BIOASSAY IN MEASURING THE EFFECTS OF AQUEOUS SOLUTIONS OF MERCURIC CHLORIDE

Determination	Response (Percent of Control) 75 µg Hg/g Soil ($HgCl_2$)
1	63
2	59
3	68
4	54
5	64
6	59
7	63
8	63
9	68
10	55
$\bar{X}$	61.6
SD	4.8

TABLE 8. DATA USED FOR DETERMINING METHOD SENSITIVITY OF THE HYDROGEN OXIDATION SOIL BIOASSAY USING AQUEOUS SOLUTIONS OF MERCURIC CHLORIDE

	Response (% of Control) µg Hg/g Soil				
Determination	10	50	75	100	150
1	79	73	63	59	38
2	92	68	59	59	41
3	85	68	68	59	38
4	72	62	54	50	41
5	80	80	64	60	38
6	79	68	59	55	35
7	79	73	63	55	38
8	79	68	63	51	35
9	79	68	68	63	38
10	79	51	55	51	41
$\bar{x}$	80.3[a]*	67.9[b]	61.6[c]	56.2[d]	38.3[e]
SD	5.1	7.5	4.8	4.4	2.2

*Mean values followed by the same letter are not significantly different at the 5% level.

the test response for concentration intervals of both 75 and 25 ppm were found to be significant at the 5 percent level. Within a mercury concentration range of 10 to 150 ppm (aqueous mercuric chloride), the Hydrogen Oxidation Soil Bioassay has a single laboratory capability of distinguishing between mercury concentration differences of 25 ppm.

LIMITS OF RELIABLE MEASUREMENT

Tests to establish the limits of reliable measurement should determine the sample concentration range for which the method is capable of providing useful data. In some instances, the single laboratory test may simply verify that the method capabilities for sensitivity, precision, and accuracy (if applicable) do not deteriorate at the upper and lower extremes of the detection range.

Upper and lower detection limits for the compounds used for the single laboratory evaluation were not available from the literature. However, data from preliminary testing showed that concentrations of most tested compounds produced extremely different effects in increments ranging between 10 and 1,000 ppm (see Figures 2-4). An effort was made to gain information on the reliability (i.e., precision and sensitivity) of the method at both of these extremes. As mentioned previously, since there was no true value or true response to a reference material, accuracy data was not obtained.

Three new concentrations of Hg were used in addition to those used for the method sensitivity test. These new concentrations were 500 ppm, 750 ppm, and 1,000 ppm. Table 9 is a compilation of test data obtained from both the additional concentrations and from the concentrations reported in Table 8. Mean test results for each sample concentration were compared using an analysis of variance as an indicator of differences in the test result. It will be noted that the method was sensitive (statistical difference at the 5 percent level) to incremental increases of Hg up to 750 ppm. Results between 750 ppm and 1,000 ppm were not statistically distinguishable. However, test results from the lower concentrations were distinguishable. Between 10 ppm and 150 ppm of Hg, the method's capability for precision can be described as having a CV range of 5.7 to 11.0 percent. Method capability for precision suffered from 500 ppm to 1,000 ppm. If the different precision capability is noted, the method might still be able to provide useful data (in terms of precision) even at the higher concentrations. The reason for an increased CV with increased Hg concentrations can probably be explained as follows. As the Hg concentration increases, the percent response (P_{2t}/P_{2c}) decreases to a point where the smaller mean values become increasingly affected by inherent deviations. The same is not true for the other extreme. As the percent response approaches the control (100 percent), only larger deviations could affect the precision. Such deviations, however, have been shown not to occur.

The limits of reliable measurement for the Hydrogen Oxidation Soil Bioassay are therefore presented as 10 ppm and 750 ppm of mercury when aqueous solutions of mercuric chloride are used as sample material.

TABLE 9. DATA USED FOR DETERMINING THE LIMITS OF RELIABLE MEASUREMENT FOR THE HYDROGEN OXIDATION SOIL BIOASSAY USING AQUEOUS SOLUTIONS OF MERCURIC CHLORIDE

	Response (% of Control) µg Hg/g Soil							
Determination	10	50	75	100	150	500	750	1000
1	79	73	63	59	38	8	2	1
2	92	68	59	59	41	10	3	2
3	85	68	68	59	38	12	2	2
4	72	62	54	50	41	11	3	1
5	80	80	64	60	38	11	3	1
6	79	68	59	55	35	10	3	1
7	79	73	63	55	38	12	3	1
8	79	68	63	51	35	10	3	1
9	79	68	68	63	38	23	3	1
10	79	51	55	51	41	8	1	1
$\bar{x}$	80.3^{a*}	67.9^{b}	61.6^{c}	56.2^{d}	38.3^{e}	11.5^{f}	2.6^{g}	1.2^{g}
SD	5.1	7.5	4.8	4.4	2.2	4.2	0.7	0.4
CV	6.4	11.0	7.8	7.8	5.7	36.5	26.9	33.3

*Mean values followed by the same letter are not significantly different at the 5% level.

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APPENDIX A

HYDROGEN OXIDATION SOIL BIOASSAY PROTOCOL

APPENDIX A

HYDROGEN OXIDATION SOIL BIOASSAY PROTOCOL

1. GENERAL DISCUSSION

Hydrogen consumption is a common soil microbiological process that can be exploited as an indicator of the effects of toxic substances in soil. An enzyme system oxidizes the hydrogen molecule (H_2) to form water (H_2O). This same system also oxidizes the radioactive form of H_2, tritium (H_2, T_2 or HT; HT will be used for this protocol) to form tritiated water (HTO). This reaction is rapid (see Figure 1) and allows for a complete bioassay to be carried out within 24 h.

This protocol contains all of the information required by a laboratory to set up and run the Hydrogen Oxidation Soil Bioassay.

2. MATERIALS

A. Heating mantle with power control for 1-L round bottom flask

B. Distilling receiver (like Corning 3622)

C. Distilling column (like Kimble 21800)

D. Heating tape (like 6 ft Briskeat rubber embedded)

E. Incubator capable of maintaining 25°C regardless of ambient temperature

F. 1-L round bottom flask (like Kimble 25285)

G. Dispenser (like Universal repipet 3020AU)

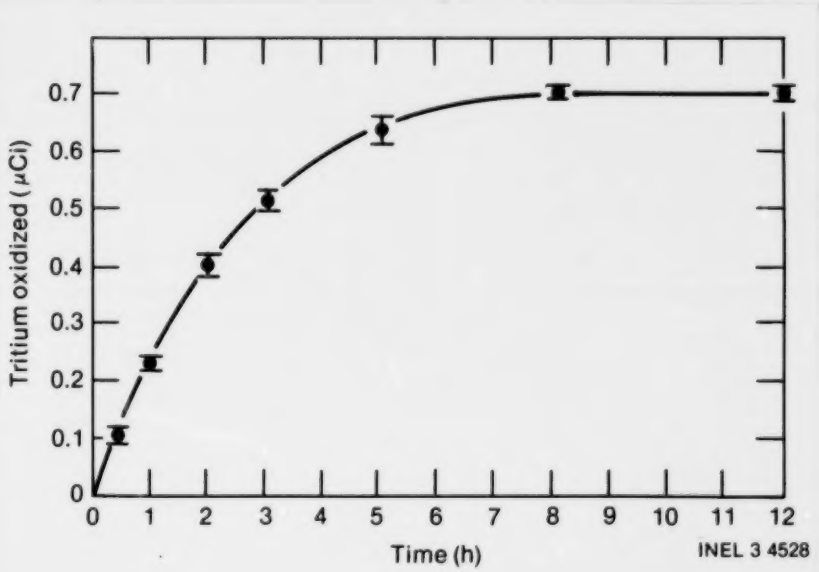

Figure 1. HT oxidation in soil with time.

H. Liquid scintillation counter

I. Gas tight syringe (like Hamilton 81620 w/Chaney adaptor)

J. Liquid scintillation vials (like VWR Scientific 66021-544)

K. 1 ea. Flowmeter (like VWR G2158-34)

L. 1 ea. General laboratory fumehood at least 4 ft by 3 ft

M. 1 ea. Top loading balance (like Sartorius 1202MP)

N. 1 box Weighing boats (like VWR 12577-049)

O. 1 ea. Micro-spoon spatula (like VWR 57952-286)

P. 1 ea. Regulator (like Matheson 3322)

Q. 2 doz #4 Rubber stoppers (like VWR 59589-198).

3. REAGENTS

A. Liquid Scintillation Counting Cocktail (like JTBaker JT9475-1)

B. Tritium (elemental) at a concentration of 5.0 mCi tritium mixed with 50 L of nitrogen (supplied by Gollub Analytical Services)

C. Soil

D. Distilled water

E. 1-g water, tritium labeled at 0.25 mCiT/g (supplied by New England Nuclear)

F. 5-L Acetone, reagent grade.

4. PROCEDURE

The general procedures for the H_2 oxidation soil bioassay are outlined in Figure 2. Detail of these various steps is provided in the following text.

A. Sample Quantity and Storage

It is recommended that the assay laboratory be provided with 1 L of each liquid sample to be tested. The laboratory should be alerted in advance so that the samples can be assayed the day they arrive. If storage is required, those samples without a degradable or volatile organic component should be stored at -4°C; otherwise store at -80°C.

B. Soil Type and Moisture Content

Soil used for the assay will be a Calico series fine sandy loam (coarse over clay mixed, thermic Aquic Xerofluent) from southeastern Nevada (53.90% sand, 10.80% clay, 1.30% organic C; 12.7 meq/100 g CEC; pH 8.6). At least 90 Kg of soil should be collected from the A1 or Ap (upper or near surface) soil horizon. After collection, the soil must be air dried and sieved before storage or use. Air drying is accomplished by spreading the soil to a thickness of up to 15 cm (with daily mixing at ambient temperature and humidity) to allow it to arrive at constant moisture content. When air dry, the soil must be sieved to pass a 0.60-mm screen. Mixing while drying and the sieving process will homogenize the soil. Finally, a determination must

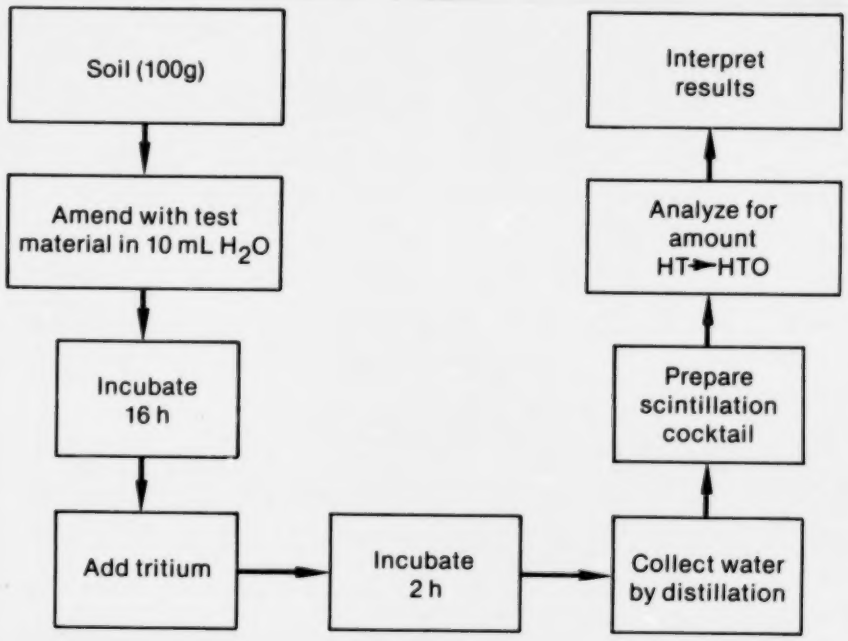

Figure 2. Outline of procedures for the Hydrogen Oxidation Soil Bioassay.

be made of the amount of water remaining in the air dried soil. This is accomplished by drying a quantity of soil in an oven at 105°C for 24 h and gravimetrically determining the amount of water loss.

The moisture-holding capacity (MHC) of this soil has been determined. (MHC is defined as the amount of water held in the soil after excess water has drained away due to gravitational force.) For the point of this protocol, the term MHC is analogous to field capacity. The quantity of water added to a dry soil at the beginning of the test is that quantity required to wet 100 g of dry, sieved soil to 25% of its MHC. That means the final water content will include the residual moisture contained after air drying, in addition to the 25% MHC. The Calico soil has a MHC of 40 ml per 100 g soil; therefore, 25% MHC is 10 ml and since this soil contained 1% moisture when it was air dried, the total water in 100 g of amended soil is 11 ml.

Basically, the test involves the use of controls (soil not treated with toxic material), positive controls (a given concentration of a toxic compound whose effect has been verified), and treatments. Both controls and treatments must be carried out in triplicate. This involves the use of three 1-L round bottom flasks per control and treatment level. Add soil (100 g) to all the required flasks before any additional step is taken.

Test compounds with which the soil is amended should be carefully prepared by mixing with water in volumetric flasks so that a final absolute concentration can be obtained. The quantity of this solution added to the 100 g of soil must be enough to bring the soil to 25% MHC. Therefore, the concentration of test compound in the volumetric flask must be of sufficient

concentration so that when some given quantity of it is added to the soil the desired concentration of chemical will be established in the soil. For example, with the Calico soil (25% MHC, 10 ml/100 g soils) 10 ml of a 10^3 ppm solution of Hg added to 100 g of soil will result in the desired 10^2 ppm concentration of Hg (100 µg Hg/g soil). Water used in this test will meet the following criteria for quality. Conductivity, less than 5 µmhos/cm^3 at 25°C; pH, 5.5-7.5; heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn) less than 0.05 mg/L; free chlorine, less than 0.1 mg/L.

C. Soil Amendment

Soil will be amended with control solutions (water for no treatment effect, 10^3 ppm solution of Hg as $HgCl_2$ for a positive control) and treatment solutions. Amendment will occur as follows. Using a 10-ml volumetric pipette, transfer either control (water and Hg solution) or test solution (treatments) to the surface of the soil inside the 1-L flask. Immediately after this addition, the soil must be stirred to ensure a uniform mixing of the added solution. Stirring will be accomplished by introducing a teflon-coated microspoon spatula (spoon end first) through the neck of the flask to the amended soil. The spatula will then be manipulated in such a manner that the wet soil surface is uniformly folded into the remaining dry soil. Mixing of the soil will be carried out at two beats per second for 30 s. At the end of this time, the flask will be stoppered and placed inside the incubator. Continue in this manner until all soil for the test has been treated. Treated soils will be incubated in the dark at 25°C for 16 h.

D. Tritium Injection

After incubation, elemental tritium will be introduced into each flask. A set of triplicated treatments (start with controls followed by flasks containing low concentration of test material to the highest) will be removed from the incubator and placed in a fume hood. These flasks will be purged one at a time with oil and water free air. This will be accomplished by removal of the rubber stopper followed with an introduction of a tube half-way into the flask. Air flow through the tube will be at 20 L/min (as indicated by a flow meter) for 10 s. The flask will then be tightly resealed. Purge the remaining two flasks. The flasks are then prepared for the introduction of elemental tritium (HT).

Commercially prepared HT will be used. A 5-ml quantity of the gas at ambient temperature and pressure must be introduced into each sealed flask. This will be accomplished using the following method. The tank holding a pressurized HT mixture will be fitted prior to the experiment with a two-stage regulator. The adjustable low pressure outlet of the regulator will be connected to a gas delivery device containing a rubber septum. A 10-ml gas tight syringe with a Chaney adaptor must be used to withdraw approximately 7 ml of HT from the delivery device. The syringe will then be removed from the delivery device and the excess gas expelled so that exactly 5 ml (as determined by the Chaney adaptor) of gas remain. This quantity of gas must immediately be introduced into the sealed flask by passing the syringe needle through the rubber stopper. The time of introduction will be recorded. After each set of triplicates has been charged, they will be returned to the incubator for an initial 2-h incubation.

E. Reaction Termination

After 2 h (to the minute), a treatment triplicate will be taken from the incubator. The rubber stoppers will be removed and the flasks purged with air as described previously. After purging, an additional 10 ml of water will be added to the soil in each flask, thus bringing the water content to 20 ml plus residal water left after air drying. The flask will then be stoppered and left at ambient temperature awaiting distillation.

F. Extraction of Water from Soil

Water will be removed from each flask by distillation. To accomplish this, a flask will be connected to a distilling receiver and column (Figure 3), which has heating tape wrapped around the arm connecting the flask to the distilling receiver. This tape should be heated to approximately 100°C during distillation. The flask must then be placed in a heating mantle. An aluminum foil cover will be placed around the exposed portion of the flask. The contents of the flask should be heated to 200°C, ±30°C. Heating will be discontinued after 15 ml of water has been collected in the distilling receiver. This water will be dispensed into a glass liquid scintillation vial. The flask will then be removed from the distillation apparatus for later cleaning. The distillation apparatus will then be cleaned by flushing with acetone and allowed to air dry. If the apparatus will not come clean with acetone, disassemble and wash with hot water and soap.

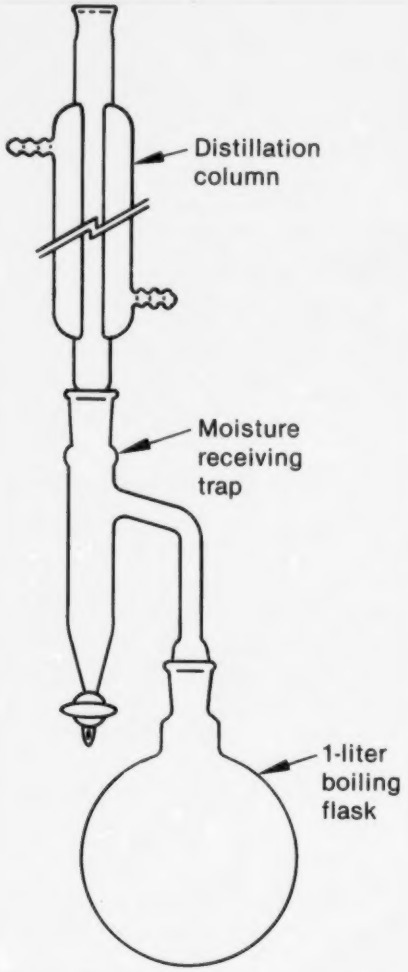

Figure 3. Distilling apparatus for the collection of tritiated water from soil exposed to tritium during the Hydrogen Oxidation Soil Bioassay.

G. Sample Counting

Water from the distillation process will be mixed with liquid scintillation cocktail as follows. Twelve millliliters of liquid scintillation cocktail will be dispensed into a 20-ml scintillation vial. To this will be added 8 ml of the distilled water. The contents will be shaken and set aside for determination of HT oxidized to HTO.

The amount of HT oxidized to HTO will be determined using a liquid scintillation counter. A HTO Standard will be kept in the machine and counted with each set of test samples. This standard will be used to determine the efficiency of the liquid scintillation counter. The term efficiency is a value calculated from the equation:

$$E = \frac{cpm}{dpm} \qquad (1)$$

where

E = efficiency scintillation counter

cpm = counts per minute as determined by counter

dpm = disintegrations per minute. There are 2.22 dpm per picocurie (pCi) (10^{-12} curie) radioactivity.

The dpm is a known value easily derived from the number of pCi of HT contained in the solution designated as the standard. For example, start with a water solution containing 0.25 mCi HT/ml or 250×10^6 pCi HT/ml. The dpm of 1 ml of this water would 555×10^6 [$(250 \times 10^6$ pCi) (222 dpm/pCi)]. If 0.5 ml of this water were diluted to 1000 ml with water, the diluted water would

contain 277.5 x 10^3 dpm/ml [(0.5 ml) (555 x 10^6 dpm) (10^{-3} dilution factor)]. Therefore, if 1 ml of this water was added to the required amount of liquid scintillation cocktail (12 ml) and the mixture brought up to 20 ml by the addition of 7 ml of distilled water, a determination of cpm could be obtained. Suppose it was determined that this mixture had an activity of 52730 cpm. By substituting into Equation (1) the calculated value E would be 0.19 or 19%.

By including this cpm standard with each sample set counted, it will be possible to determine whether machine efficiency is constant with time (a QA check) and make it possible to convert cpm values of samples into dpm for further calculations.

Because liquid scintillation is based on analytical recognition of photon (light) production resulting from radioactive decay, it is possible to have results distorted due to a phenomenon known as quenching. Quenching happens whenever the material placed into a liquid scintillation cocktail prevents the measurement of photons. Among other things, this can occur due to photon absorption. Fortunately, once the amount of quenching is calculated it is a simple matter to make adjustments in order to obtain unbiased analytical counting data. In the context of the bioassay, there will be times when distillates from soil treated with organics will be moderately to severely colored. The possibility of quenching occurring in such samples can be easily determined by the following procedure. After an initial analysis, spike these samples with a known concentration of HTO (0.1 µCi HTO/0.1 ml H_2O). It is possible to calculate the cpm this additional HT will produce in an unquenched solution (Equation 1). The percent quenching is then calculated from the equation:

$$Q = [1-(\frac{cpms}{cpmc})]\ 100 \qquad (2)$$

where

Q = percent quenching

cpms = increase cpm obtained by spiking sample

cpmc = calculated cpm increase.

If quenching does occur, subsequent data should be normalized using the Q value.

5. CALCULATIONS

A. Tabulation of Data

Data will be recorded and reported on a data worksheet shown in Figure 4.

B. Data Transformation

Data for each section of the worksheet is calculated in the following manner:

1. Average cpm: This is the mean value for the three replications of each treatment.

2. Coefficient of variation (CV): This value is obtained by dividing the replicate standard deviation (σ) by the replicate mean ($\bar{X}$).

$$CV = \sigma/\bar{X}. \qquad (3)$$

HYDROGEN OXIDATION WORK SHEET

μCi HT Injected _____ Test Compound _____

Soil Series _____

Conc of Test Compound	Ave cpm (3 replicates)	CV	Ave dpm	Y	P2	% of Control

Figure 4. Worksheet used to record data from the Hydrogen Oxidation Bioassay.

3. Average dpm: The average cpm value converted into dpm. Recall Equation 1: E = cpm/dpm and solve for dpm or

$$dpm = cpm/E. \quad (4)$$

4. Amount of HT oxidized (Y) reported as microcuries (μCi): Recall there are 2.22 dpm per pCi HT and that 1 pCi is 10^{-6} μCi, then

$$Y = (2.22)(dpm\ [Equation\ 4])(10^{-6}). \quad (5)$$

5. Reaction rate parameter (P_2):

$$P_2 = \frac{[\ln (1-Y/P_1)]}{-t} \quad (6)$$

where

Y = amount of HT oxidized in μCi (Equation 5)

P_1 = initial amount of HT injected in μCi

t = time soil was incubated with HT (h)

ln = natural logarithm.

6. Percent of reaction rate of treatments compared to control:

$$\% = P_{2t}/P_{2c} \quad (7)$$

where

P_{2t} = P_2 of treatment

P_{2c} = P_2 of control.

C. Data Interpretation

The percent reaction rates (Equation 7) for each treatment are plotted against log concentration of the test compound as seen in Figure 5. From the plot it will be possible to determine what concentration of test compound reduces P_2 by 50% ($P_2$50). This value will serve as an index with which to compare toxicity of compounds. The positive control will be used as an intercomparison.

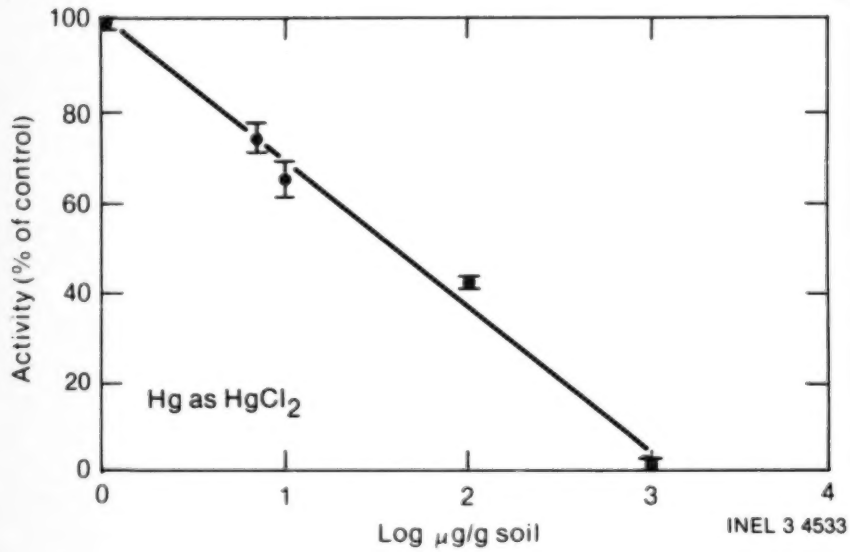

Figure 5. Example of % reaction rates plotted to determine the effect of toxicants using the Hydrogen Oxidation Bioassay.

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APPENDIX B

ANALYTICAL RESULTS FOR SAMPLE MATERIAL USED DURING THE EVALUATION

Analytical Summary

Organic and inorganic confirmation analyses were conducted on sample material used during the single laboratory evaluation. This summary includes analytical results for: 1) three soil samples, 2) the respective stock solutions of mercuric chloride ($HgCl_2$) and m-chlorophenol (mcp), and 3) several dilutions of the $HgCl_2$ and mcp stock material (Appendix Table I). The analyses were conducted by Acurex Corporation (Energy and Environmental Division).

Organic analyses (EPA Method 625.1) were conducted following extraction with methylene chloride. Gas chromatographic/mass spectrometric analyses were performed using a Finnigan 4021 GC/MS and an Incos 2300 data system. Surrogate compounds used for the soil samples included 2-fluorobiphenyl (100 µg/sample), nitrobenzene-d_5 (600 µg/sample), pyrene-d_{10} (100 µg/sample), and terphenyl-d_{14} (100 µg/sample). The surrogate compound used for the m-chlorophenol samples was p-chlorophenol-d_4, which was added at between 100 µg and 10,000 µg based on the volume of final extract.

Mercury analyses were conducted using atomic absorption spectroscopy (Official Methods of Analysis, AOAC, 1980). Inductively coupled argon plasma spectroscopy (ICAP) was used to screen the samples for additional inorganics.

Analytical results are given in Appendix Tables II, III, and IV.

APPENDIX TABLE I

SUMMARY OF CHEMICAL ANALYSES PERFORMED BY ACUREX CORPORATION ON SAMPLE MATERIAL USED DURING SINGLE LABORATORY EVALUATION OF THE HYDROGEN OXIDATION SOIL BIOASSAY TECHNIQUE

Analytical Laboratory Identification	Description of Material Requiring Confirmation Analysis	Type of Analysis		
		GC/MS for Organics	ICAP	AA for Hg
-1	200 g untreated calico soil	X	X	X
-2	200 g soil amended with $HgCl_2$ (10^3 µg Hg/g soil)	X	X	X
-3	200 g soil amended with m-chlorophenol (10^3 µg mcp/g soil)	X	X	X
-4	20 mL mcp stock (10^4ppm)	X	X	X
-5	10 mL $HgCl_2$ stock (10^4ppm Hg)*		X	X
-6	20 mL mcp 10^3 ppm (dilutions from stock)	X		
-7	20 mL mcp 10^2 ppm (dilutions from stock)	X		
-8	20 mL mcp 10 ppm (dilutions from stock)	X		
-9	10 mL $HgCl_2$ 5 x 10^3 ppm Hg			X
-10	10 mL $HgCl_2$ 10^3 ppm Hg			X
-11	10 mL $HgCl_2$ 750 ppm Hg (dilutions from stock)			X
-12	10 mL $HgCl_2$ 500 ppm Hg (dilutions from stock)			X
-13	10 mL $HgCl_2$ 100 ppm Hg (dilutions from stock)			X

*Organic analysis was not performed because there was not enough sample to extract.
m-chlorophenol (mcp)
mercuric chloride ($HgCl_2$)

APPENDIX TABLE II

ACUREX CORPORATION ANALYTICAL RESULTS FOR
SINGLE LABORATORY TEST SAMPLES CONTAINING m-CHLOROPHENOL

Description of Material	Analytical Laboratory Identification	Concentration (ppm)		Recovery of p-Chlorophenol-d_4
		Uncorrected for Recovery	Corrected for Recovery	
mcp stock (10^4 ppm)	-4	9,100	11,400	80
mcp 10^3 ppm	-6	965	965	100
mcp 10^2 ppm	-7	109	86	127
mcp 10 ppm	-8	7.4	9.9	75
mcp 10 ppm	-8	6.35	9.9	64

APPENDIX TABLE III

INORGANIC ANALYSES PERFORMED BY ACUREX CORPORATION ON MATERIAL USED DURING SINGLE LABORATORY EVALUATION OF THE HYDROGEN OXIDATION SOIL BIOASSAY TECHNIQUE

	Concentration (ppm)									
Description of Material	Untreated Calico Soil	Soil Amended with $HgCl_2$	Soil Amended with mcp	mcp Stock (10^4ppm)	$HgCl_2$ Stock (10^4ppm)	$HgCl_2$ (5000ppm)	$HgCl_2$ (1000ppm)	$HgCl_2$ (750ppm)	$HgCl_2$ (500ppm)	$HgCl_2$ 100ppm)
Analytical Laboratory ID	-1	-2	-3	-4	-5	-9[a]	-10[a]	-11[a]	-12[a]	-13[a]
As	<1.0	<1.0	1.1	<0.1	<0.1					
Sb	<100	<100	<100	<1.0	<1.0					
Ba	<100	<100	<100	<10	<10					
Be	<1.0	<1.0	<1.0	<0.05	<0.05					
Cd	<1.0	<1.0	<1.0	<0.1	<0.1					
Co	<80	<80	<80	<1.0	<1.0					
Cu	<15	<15	<15	<1.0	<1.0					
Pb	<5.0	<5.0	<5.0	<1.0	<1.00					
Hg	<0.2	830	<0.2	<0.05	9400	4500	990	700	480	97
Mo	<350	<350	<350	<2.0	<2.0					
Ni	<20	<20	<20	<1.0	<1.0					
Se	<1.0	<1.0	<1.0	<0.5	<0.5					
Ag	<5.0	<5.0	<5.0	<1.0	<1.0					
Tl	<5.0	<5.0	<5.0	<1.0	<1.0					
Va	<24	<24	<24	<1.0	<1.0					
Zn	25	27	25	<1.0	<1.0					
Cr	<5.0	<5.0	<5.0	<1.0	<1.0					

a - Analyzed only for Hg

APPENDIX TABLE IV

ORGANIC ANALYSES PERFORMED BY ACUREX CORPORATION
ON TREATED AND UNTREATED SOIL USED DURING SINGLE LABORATORY
EVALUATION OF THE HYDROGEN OXIDATION SOIL BIOASSAY TECHNIQUE

	Concentration (µg/Kg)		
	Untreated Calico Soil	Soil Amended with $HgCl_2$	Soil Amended with mcp*
Compound			
Naphthalene	440	ND	ND; ND
Unidentified alkanes	P	P	P; P
m-Chlorophenol	ND	ND	27,300; 150,000
Detection Limit	50	50	50; 50
Percent Surrogate Recoveries			
2-Fluorobiphenyl	74	68	123; 83
Nitrobenzene-d_5	63	80	81; 56
Pyrene-d_{10}	92	92	105; 90
Terphenyl-d_{12}	64	86	101; 83
Decafluorobiphenyl	36	22	64; 32

ND - Not detected

P - Present but not quantitated

* Material analyzed in duplicate. Results indicate that mcp may not be distributed homogeneously in the matrix.